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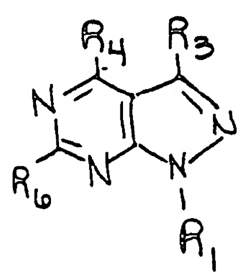


(I)

(57) Abstract

This invention is directed to novel 3,4-disubstituted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines and to the use of these compounds in the preparation of oligonucleotides. The invention is also directed to nucleosides and mono- and oligonucleotides comprising at least one of these pyrazolopyrimidines, and to the use of the resulting novel oligonucleotides for diagnostic purposes. More particularly, the pyrazolopyrimidines of the present invention are of formula (I), wherein R_1 is hydrogen, or a sugar moiety optionally substituted at its 3′ or its 5′ position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen, then R_1 cannot be hydrogen; R_3 is hydrogen or the group $-W-(X)_n-A$; each of W and X is independently a chemical linker arm; A is an intercalator, an electrophilic crosslinker or a reporter group; each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$; R is H or C_{1-6}alkyl; n is zero or one; and t is zero to twelve. The novel oligo- and polynucleotides are useful in the identification, isolation, localization and/or detection of complementary nucleic acid sequences of interest in cell-free or cellular systems. Therefore, the invention further provides a method for identifying target nucleic acid sequences, which method comprises utilizing an oligo- or polynucleotide probe comprising at least one of a labeled pyrazolo[3,4-d]pyrimidine of the present invention.

DERIVATIVES OF PYRAZOLO[3,4-d]PYRIMIDINE

BACKGROUND OF THE INVENTION

This invention relates to derivatives of pyrazolo- [3,4-d]pyrimidine and to the use of these compounds for the preparation of oligonucleotides.

Oligonucleotides are useful as diagnostic probes for the detection of "target" DNA or RNA sequences. In the past, such probes were made up of sequences of nucleic acid containing purine, pyrimidine or 7-deazapurine nucleotide bases (U.S. Patent 4,711,955). The method for attaching chemical moieties to these bases has been via an acetoxy- mercuration reaction, which introduces covalently bound mercury atoms into the 5-position of the pyrimidine ring, the C-8 position of the purine ring or the C-7 position of a 7-deazapurine ring (Dale et al., Proc. Natl. Acad. Sci. USA 70:2238 (1973); Dale et al., Biochemistry 14:2447 (1975)), or by the reaction of organomercurial compounds with olefinic compounds in the presence of palladium catalysts (Ruth et al., J. Org. Chem. 43:2870 (1978); Bergstrom et al., J. Am. Chem. Soc. 100:8106 (1978); Bigge et al., J. Am. Chem. Soc. 102:2033 (1980)).

The sugar component of oligonucleotide probes has been, until the present, composed of nucleic acid containing ribose or deoxyribose or, in one case, natural β-arabinose (patent publication EP 227,459).

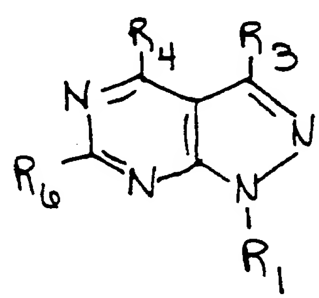
A novel class of nucleotide base, the 3,4-disubst ituted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines, has now been found which offers several advantages over the prior art. The *de novo* chemical synthesis of the pyrazolopyrimidine and the resulting nucleotide allows for the incorporation of a wide range of functional groups in a variety of

different positions on the nucleotide base and for the use of different sugar moieties. Also, adenine, guanine and hypoxanthine analogs are obtain d from a single nucleoside precursor. Additionally, the synthesis does not require the use of toxic heavy metals or expensive catalysts. Similar pyrazolo[3,4-d]pyrimidines are known (Kobayashi, Chem. Pharm. Bull. 21:941 (1973)); however, the substituents on the group are different from those of the present invention and their only use is as pharmaceuticals, more particularly as xanthine oxidase inhibitors.

SUMMARY OF THE INVENTION

This invention is directed to novel 3,4-disubsti- tuted and 3,4,6-trisubstituted pyrazolo[3,4-d]pyrimidines and to the use of these compounds in the preparation of oligonucleotides. The invention is also directed to nucleosides and mono- and oligonucleotides comprising at least one of these pyrazolopyrimidines, and to the use of the resulting novel oligonucleotides for diagnostic purposes.

More particularly, the pyrazolopyrimidines of the present invention are of the following formula (I):



(I)

wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen, then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker, a photoactivatable crosslinker, or a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl;

n is zero or one; and

t is zero to twelve.

The invention also provides novel nucleosides and nucleotides comprising at least one of the above pyrazolo- pyrimidines.

Nucleotides of this invention and oligo- and polynucleotides into which the nucleotides have been incorporated may be used as probes. As such, the novel oligo- and polynucleotides are useful in the identification, isolation, localization and/or detection of complementary nucleic acid sequences of interest in cell-free or cellular systems. Therefore, the invention further provides a method for identifying target nucleic acid sequences, which method comprises utilizing an oligo- or polynucleotide probe comprising at least one of a labeled pyrazolo[3,4-d]pyrimi- dine of the present invention.

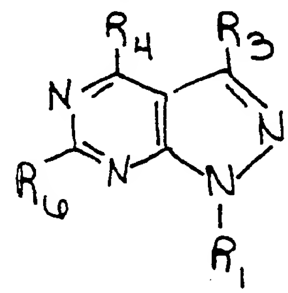
DETAILED DESCRIPTION OF THE INVENTION

This invention provides novel substituted pyrazolo[3,4-d]pyrimidines which are used as the nucleotide base in preparing nucleosides and nucleotides, rather than the natural purine or pyrimidine bases or the deazapurine analogs.

The synthesis of 3,4-disubstituted and 3,4,6-tri- substituted pyrazolo[3,4-d]pyrimidine nucleosides and their use as reagents for incorporation into nucleic acids either enzymatically or via chemical synthesis offers several advantages over current

procedures. The de novo chemical synthesis of the nucleotide allows for th incorporation of a wide rang of functional groups (e.g., NH_2, SH, OH, halogen, COOH, CN, $CONH_2$) and the use of different sugar moieties. Also, adenine, guanine, and hypoxanthine analogs are obtained from a single nucleoside precursor. And, the synthesis does not require the use of toxic heavy metals or expensive catalysts.

More particularly, the pyrazolopyrimidines of the present invention are of the following formula (I):



(I)

wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen, then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl;

n is zero or one; and

t is zero to twelve.

The sugar moiety is selected from those useful as a component of a nucleotide. Such a moiety may be selected from, for example, ribose, deoxyribose, glucose, arabinose, xylose and lyxose. The sugar

moiety is preferably ribose, deoxyribose or arabinose and embraces either anomer, α or β.

A reactive group suitable for internucleotide bond formation is one which is useful during chain extension in the synthesis of an oligonucleotide. Reactive groups particularly useful in the present invention are those containing phosphorus. Phosphorus-containing groups suitable for internucleotide bond formation are preferably alkyl phosphorchloridites or alkylphosphoramidites. Alternatively, activated phosphate diesters may be employed for this purpose.

A chemical linker arm (W alone or together with X) serves to make the functional group (A) more able to readily interact with antibodies, detector proteins, or chemical reagents, for example. The linkage holds the functional group away from the base when the base is paired with another within the double-stranded complex. Linker arms may include alkylene groups of 1 to 12 carbon atoms, alkenylene groups of 2 to 12 carbon atoms and 1 or 2 olefinic bonds, alkynylene groups of 2 to 12 carbon atoms and 1 or 2 acetylenic bonds, or such groups substituted at a terminal point with nucleophilic groups such as oxy, thio, amino or chemically blocked derivatives thereof (e.g., trifluoroacetamido). Such functionalities, including aliphatic or aromatic amines, exhibit nucleophilic properties and are capable of serving as a point of attachment of the functional group (A).

The linker arm moiety (W alone or together with X) is preferably of at least three atoms and more preferably of at least five atoms. The terminal nucleophilic group is preferably amino or chemically blocked derivatives thereof (e.g., trifluoroacetamido).

Intercalators are planar aromatic bi-, tri- or polycyclic molecules whose dimensions are roughly the same as those of a purine-pyrimidine pair and which

can insert themselves between two adjacent base pairs in a double stranded helix of nucleic acid. Intercalators have been used to caus frameshift mutations in DNA and RNA. It has also recently been shown that when an intercalator is covalently bound via a linker arm ("tethered") to the end of a deoxyoligonucleotide, it increases the binding affinity of the oligonucleotide for its target sequence, resulting in strongly enhanced stability of the complementary sequence complex. At least some of the tethered intercalators also protect the oligonucleotide against exonucleases, but not against endonucleases. See, Sun et al., Nucleic Acids Res. 15:6149-6158 (1987); Le Doan et al., Nucleic Acids Res. 15:7749-7760 (1987). Examples of tetherable intercalating agents are oxazolopyridocarbazole, acridine orange, proflavine, acriflavine and derivatives of proflavine and acridine such as 3-azido-6-(3-bromopropylamino)acridine, 3-amino-6-(3-bromopentylamino)acridine, and 3-methoxy-6- chloro-9-(5-hydroxypentylamino)acridine.

Oligonucleotides capable of crosslinking to the complementary sequence of target nucleic acids are valuable in chemotherapy because they increase the efficiency of inhibition of mRNA translation or gene expression control by covalent attachment of the oligonucleotide to the target sequence. This can be accomplished by crosslinking agents being covalently attached to the oligonucleotide, which can then be chemically activated to form crosslinkages which can then induce chain breaks in the target complementary sequence, thus inducing irreversible damage in the sequence. Examples of electrophilic crosslinking moieties include alpha-halocarbonyl compounds, 2-chloroethylamines and epoxides.

When oligonucleotides comprising at least one nucleotide base moiety of the invention are utilized as

a probe in nucleic acid assays, a label is attached to detect the presenc of hybrid polynucleotides. Such labels act as reporter groups and act as means for detecting duplex formati n b tween the target nucleotides and their complementary oligonucleotide probes.

A reporter group as used herein is a group which has a physical or chemical characteristic which can be measured or detected. Detectability may be provided by such characteristics as color change, luminescence, fluorescence, or radioactivity; or it may be provided by the ability of the reporter group to serve as a ligand recognition site.

Probes may be labeled by any one of several methods typically used in the art. A common method of detection is the use of autoradiography with ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P labeled probes or the like. Other reporter groups include ligands which bind to antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents, enzymes and enzyme substrates. Alternatively, the same components may be indirectly bonded through a ligand-antiligand complex, such as antibodies reactive with a ligand conjugated with label. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The choice of label dictates the manner in which the label is incorporated into the probe. Radioactive probes are typically made using commercially available nucleotides containing the desired radioactive isotope. The radioactive nucleotides can be incorporated into probes, for example, by using DNA synthesizers, by nick-translation, by tailing of radioactive bases to the 3' end of probes with terminal transferase, by

copying M13 plasmids having specific inserts with the Klenow fragment of DNA polymerase in the presence of radioactive dNTP's, or by transcribing RNA from templates using RNA polymerase in the presence of radioactive rNTP's.

Non-radioactive probes can be labeled directly with a signal (e.g., fluorophore, chemiluminescent agent or enzyme) or labeled indirectly by conjugation with a ligand. For example, a ligand molecule is covalently bound to the probe. This ligand then binds to a receptor molecule which is either inherently detectable or covalently bound to a detectable signal, such as an enzyme or photoreactive compound. Ligands and antiligands may be varied widely. Where a ligand has a natural "antiligand", namely ligands such as biotin, thyroxine, and cortisol, it can be used in conjunction with its labeled, naturally occurring antiligand. Alternatively, any haptenic or antigenic compound can be used in combination with a suitably labeled antibody. A preferred labeling method utilizes biotin-labeled analogs of oligonucleotides, as disclosed in P. Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981), which is incorporated herein by reference.

Enzymes of interest as reporter groups will primarily be hydrolases, particularly phosphatases, esterases, ureases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescers include luciferin, and 2,3-dihydrophthalazinediones, e.g., luminol.

The specific hybridization conditions are not critical and will vary in accordance with the investigators preferences and needs. Various hybridization solutions may be employed, comprising fr m about 20 to 60% volume, preferably 30%, of a polar

organic solvent. A common hybridization solution employs about 30-60% v/v formamide, about 0.5 to 1M sodium chloride, about 0.05 to 0.1M buffers, such as sodium citrate, Tris HCl, PIPES or HEPES, about 0.05 to 0.5% detergent, such as sodium dodecylsulfate, and between 1-10 mM EDTA, 0.01 to 5% ficoll (about 300-500 kilodaltons), 0.1 to 5% polyvinylpyrrolidone (about 250-500 kdal), and 0.01 to 10% bovine serum albumin. Also included in the typical hybridization solution will be unlabelled carrier nucleic acids from about 0.1 to 5 mg/ml, e.g., partially fragmented calf thymus or salmon sperm, DNA, and/or partially fragmented yeast RNA and optionally from about 0.5 to 2% wt./vol. glycine. Other additives may also be included, such as volume exclusion agents which include a variety of polar water-soluble or swellable agents, such as anionic polyacrylate or polymethylacrylate, and charged saccharidic polymers, such as dextran sulfate.

The particular hybridization technique is not essential to the invention. Hybridization techniques are generally described in Nucleic Acid Hybridization, A Practical Approach, Ed. Hames, B.D. and Higgins, S.J., IRL Press, 1985; Gall and Pardue (1969), Proc. Natl. Acad. Sci., U.S.A., 63:378-383, and John, Burnsteil and Jones (1969) Nature, 223:582-587. As improvements are made in hybridization techniques, they can readily be applied.

The amount of labelled probe which is present in the hybridization solution may vary widely. Generally, substantial excesses of probe over the stoichiometric amount of the target nucleic acid will be employed to enhance the rate of binding of the probe to the target DNA.

Various degrees of stringency of hybridization can be employed. As the conditions for hybridization become more stringent, there must be a greater degree of complementarity between the probe and

the target for the formation of a stable duplex. The d gree of stringency can be controlled by temperature, ionic strength, the inclusion of polar organic solvents, and the like. For example, temperatures employed will normally be in the range of about 20 to 80 C, usually 25 to 75 C. For probes of 15- 50 nucleotides in 50% formamide, the optimal temperature range can vary from 22-65°C. With routine experimentation, one can define conditions which permit satisfactory hybridization at room temperature. The stringency of hybridization is also conveniently varied by changing the ionic strength and polarity of the reactant solution through manipulation of the concentration of formamide within the range of 20% to 50%.

Treatment with ultrasound by immersion of the reaction vessel into commercially available sonication baths can often times accelerate the hybridization rates.

After hybridization at a temperature and time period appropriate for the particular hybridization solution used, the glass, plastic, or filter support to which the probe-target hybrid is attached is introduced into a wash solution typically containing similar reagents (e.g., sodium chloride, buffers, organic solvents and detergent), as provided in the hybridization solution. These reagents may be at similar concentrations as the hybridization medium, but often they are at lower concentrations when more stringent washing conditions are desired. The time period for which the support is maintained in the wash solutions may vary from minutes to several hours or more.

Either the hybridization or the wash medium can be stringent. After appropriate stringent washing, the correct hybridization complex may now be detected in accordance with the nature of the label.

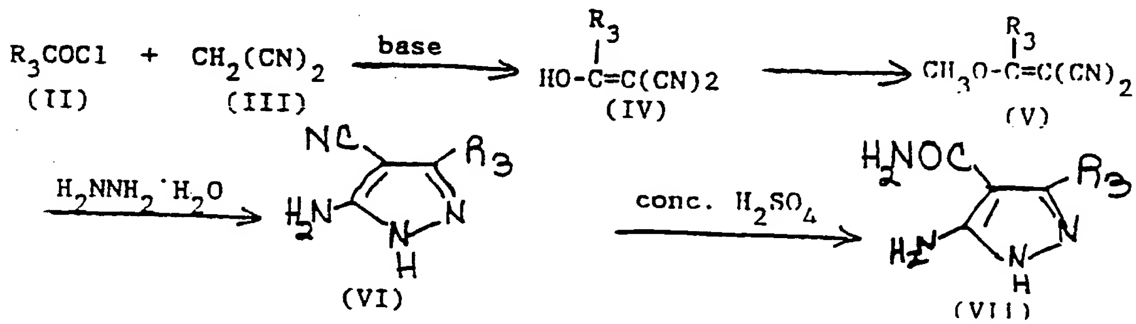
The probe may be conjugat d directly with the label. For example, where the label is radioactive, the support surface with associated hybridization complex substrate is exposed to X-ray film. Where the label is fluorescent, the sample is detected by first irradiating it with light of a particular wavelength. The sample absorbs this light and then emits light of a different wavelength which is picked up by a detector (Physical Biochemistry, Freifelder, D., W.H. Freeman & Co., 1982, pp. 537-542). Where the label is an enzyme, the sample is detected by incubation an appropriate substrate for the enzyme. The signal generated may be a colored precipitate, a colored or fluorescent soluble material, or photons generated by bioluminescence or chemi-luminescence. The preferred label for dipstick assays generates a colored precipitate to indicate a positive reading. For example, alkaline phosphatase will dephosphorylate indoxyl phosphate which then will participate in a reduction reaction to convert tetrazolium salts to highly colored and insoluble formazans.

Detection of a hybridization complex may require the binding of a signal generating complex to a duplex of target and probe polynucleotides or nucleic acids. Typically, such binding occurs through ligand and anti-ligand interactions as between a ligand-conjugated probe and an anti-ligand conjugated with a signal. The binding of the signal generation complex is also readily amenable to accelerations by exposure to ultrasonic energy.

The label may also allow indirect detection of the hybridization complex. For example, where the label is a hapten or antigen, the sample can be detected by using antibodies. In these systems, a signal is generated by attaching fluorescent or enzyme molecules to the antibodies or in some cases, by attachment to a radioactive label. (Tijssen, P.,

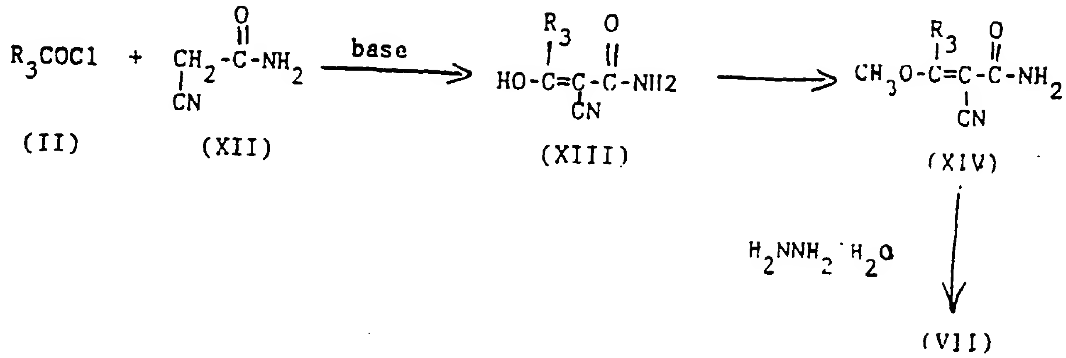
Practice and Theory of Enzyme Immunoassays, Laboratory Techniques in Biochemistry and Molecular Biology, Burdon, R.H., van Knippenberg, P.H., Eds., Elsevier, 1985, pp. 9-20).

The compounds of the present invention of formula I where R_1 is hydrogen may be prepared by the procedures outlined below and as set forth by Kobayashi in Chem. Pharm. Bull. 21:941-951 (1973), the disclosure of which is incorporated herein by reference.

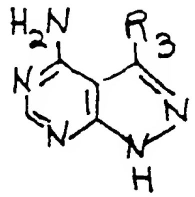


In general, malononitrile (III) is treated with acyl halide (II) in the presence of a base to yield acylmalononitrile (IV), which is subsequently methylated with dimethyl sulfate or diazomethane, for example, to give the substituted methoxymethylenemalononitrile (V). This compound is then reacted with hydrazine hydrate in boiling alcohol to give the 3-substituted-5-aminopyrazole-4-carbonitrile (VI), which is treated with cold concentrated sulfuric acid to give the 3-substituted-5-aminopyrazole-4-carboxamide (VII).

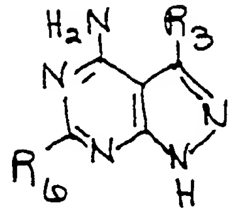
The carboxamide (VII) may alternatively be prepared by treating cyanoacetamide (XII) with acid halide (II) to give the acylcyanoacetamide (XIII), which is then methylated and the resulting methoxy compound (XIV) is reacted with hydrazine hydrate.



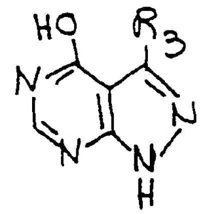
Syntheses of pyrazolo[3,4-d]pyrimidines are accomplished from the two pyrazole intermediates, VI and VII. Thus, 3,4-disubstituted pyrazolo[3,4-d]pyrimidines (VIII and X) are obtained by treating the corresponding VI and VII with boiling formamide. Alternatively, VI may be treated with dialkoxymethyl ester of a carboxylic acid, at room temperature or above room temperature, and then with ammonia to give VIII, and VII may be treated with dialkoxymethyl ester of a carboxylic acid (without subsequent ammonia treatment), at room temperature or above room temperature, to give X. 3,4,6-Trisubstituted pyrazolo- [3,4-d]pyrimidines (IX and XI) are obtained by fusing the corresponding VI and VII with urea and thiourea $(H_2N)_2C=R_6$ (where R_6 is O or S). Alternatively, VI and VII may be treated with an alkyl xanthate salt such as potassium ethyl xanthate and with alkyl halide such as methyl iodide, at a temperature above room temperature, followed by oxidation by a peroxide such as m-chloroperbenzoic acid (MCPBA) and subsequent treatment with ammonia to give IX and XI, respectively, where R_6 is NH_2.



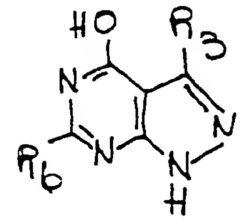
(VIII)



(IX)



(X)



(XI)

The compounds of formula I may be recovered from the reaction mixture in which they are formed by established procedures.

In the compounds of formula I where R_1 is a sugar moiety, the sugar may be either added to the 1-position of the pyrazole VI or VII prior to further treatment or added to the 1-position of the pyrazolo[3,4-d]pyrimidine VIII, IX, X or XI. To add the sugar, the pyrazole or pyrazolopyrimidine is treated with sodium hydride and then with the glycosyl halide of the blocked sugar.

Oligonucleotides of the present invention comprise at least one and up to all of their nucleotides from the substituted pyrazolo[3,4-d]pyrimidines of formula I.

To prepare oligonucleotides, protective groups are introduced onto the nucleosides of formula I and the nucleosides are activated for use in the synthesis of oligonucleotides. The conversion to protected, activated forms follows the procedures as described for 2'-deoxynucleosides in detail in several reviews. See, Sonveaux, Bioorganic Chemistry 14:274-325 (1986); Jones, in *Oligonucleotide Synthesis, a Practical Approach*, M.J. Gait, Ed., IRL Press, p. 23-34 (1984).

The activated nucleotides are incorporated into oligonucleotides in a manner analogous to that for DNA and RNA nucleotides, in that the correct nucleotides will be sequentially linked to form a chain

of nucleotides which is complementary to a sequence of nucl otides in target DNA or RNA. The nucleotides may be incorporated either enzymatically or via chemical synthesis. In a preferred embodiment, the activated nucleotides may substitute for an adenine using the nick translation procedure, as described by Langer et al., Proc. Natl. Acad. Sci. USA 78:6633-6637 (1981), the disclosure of which is incorporated herein by reference. In another preferred embodiment, the activated nucleotides may be used directly on an automated DNA synthesizer according to the procedures and instructions of the particular synthesizer employed. The oligonucleotides may be prepared on the synthesizer using the standard commercial phosphoramidite or H-phosphonate chemistries.

An oligonucleotide probe according to the invention includes at least one labeled substituted pyrazolo[3,4-d]pyrimidine nucleotide moiety of formula I.

The amount of labeled probe present in the hybridization solution may vary widely, depending upon the nature of the label, the amount of the labeled probe that can reasonably bind to the cellular target nucleic acid, and the precise stringency of the hybridization medium and/or wash medium. Generally, substantial probe excesses over the stoichiometric amount of the target will be employed to enhance the rate of binding of the probe to the target nucleic acids.

The invention is also directed to a method for identifying target nucleic acid sequences, which method comprises utilizing an oligonucleotide probe including at least one labeled substituted pyrazolo[3,4-d]pyrimidine nucleotide moiety of formula I.

In one embodiment, the method comprises the steps of:

(a) denaturing nucleic acids in the sample to be tested;

(b) hybridizing to the target nucleic acids an oligonucleotide probe including at least one labeled substituted pyrazolo[3,4-d]pyrimidine, wherein the probe comprises a sequence complementary to that of the target nucleic acids;

(c) washing the sample to remove unbound probe;

(d) incubating the sample with detection agents; and

(e) inspecting the sample.

The above method may be conducted following procedures well known in the art.

An assay for identifying target nucleic acid sequences utilizing an oligonucleotide probe including at least one labeled substituted pyrazolo[3,4-d]pyrimidine nucleotide moiety of formula I and comprising the above method is contemplated for carrying out the invention. Such an assay may be provided in kit form. For example, a typical kit will include a probe reagent component comprising an oligonucletide including at least one labeled pyrazolo[3,4-d]pyrimidine, the oligonucleotide having a sequence complementary to that of the target nucleic acids; a denaturation reagent for converting double-stranded nucleic acid to single-stranded nucleic acid; and a hybridization reaction mixture. The kit can also include a signal-generating system, such as an enzyme for example, and a substrate for the system.

The following examples are provided to illustrate the present invention without limiting same. "RT" means room temperature.

EXAMPLE 1:

6-(Tritylamino)caproic Acid.

6-Aminocaproic acid (26 g, 0.2 mmole) was dissolved in dichloromethane (200 mL) by the addition of triethylamine (100 mL). Trityl chloride (120 g, 0.45 mmole) was added and the solution stirred for 36h. The resulting solution was extracted with 1N HCl and the organic layer evaporated to dryness. The residue was suspended in 2-propanol/1N NaOH (300 mL/100 mL) and refluxed for 3h. The solution was evaporated to a thick syrup and added to dichloromethane (500 mL). Water was added and acidified. The phases were separated, the organic layer dried over sodium sulfate, and evaporated to dryness. The residue was suspended in hot 2-propanol, cooled, and filtered to give 43.5 g (58%) of 6-(tritylamino)caproic acid, useful as an intermediate compound.

EXAMPLE 2:

5-(Tritylamino)pentylhydroxymethylenemalononitrile.

To a dichloromethane solution of 6-(tritylamino)caproic acid (20.0 g, 53 mmole) and triethylamine (20 mL) in an ice bath was added dropwise over 30 min *i*-butylchloroformate (8.3 mL, 64 mmole). After stirring for 2 hr in an ice bath, freshly distilled malononitrile (4.2 g, 64 mmole) was added all at once. The solution was stirred for 2 hr in an ice bath and for 2 hr at RT. The dichloromethane solution was washed with ice cold 2N HCl (300 mL) and the biphasic mixture filtered to remove product that precipitates (13.2 g). The phases were separated and the organic layer dried and evaporated to a thick syrup. The syrup was covered with dichloromethane and on standing deposited fine crystals of product. The crystals were filtered and dried to give 6.3 g for a

total yield of 19.5 g (87%) of the product, which is useful as an intermediate.

EXAMPLE 3:

5-(Tritylamino)pentylmethoxymethylenemalononitrile.

A suspension of the malononitrile of Example 2 (13 g, 31 mmole) in ether/dichloromethane (900 mL/100 mL), cooled in an ice bath, was treated with a freshly prepared ethereal solution of diazomethane (from 50 mmole of Diazald/ (Aldrich Chemical Company)). The solution was stirred for 6 hr and then neutralized with acetic acid (10 mL). The solution was evaporated to dryness and the residue chromatographed on silica gel using dichlormethane/acetone (4/1) as the eluent. Fractions containing product were pooled and evaporated to a syrup. The syrup was triturated with dichloromethane to induce crystallization. The crystals were filtered and dried to give 8.3 g (61%) of chromatographically pure product, useful as an intermediate compound.

EXAMPLE 4:

5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.

To a methanol solution (100 mL) of the product of Example 3 (7.0 g, 16 mmole) in an ice bath was added hydrazine monohydrate (7.8 mL, 160 mmole) dropwise over 15 min. After stirring for 30 min in an ice bath, the solution was evaporated to dryness. The residue was suspended in cold methanol and filtered to give 7.1 g (100%) of 5-amino-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile, useful as an intermediate, after drying. An analytical sample was prepared by recrystallization from water.

EXAMPLE 5:

5-Amino-1-(2-deoxy-3,5-di-O-toluoyl-β-D-erythropentofu-ranos-yl)-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile.

An ice cold solution of the carbonitrile from Example 4 (3.5 g, 8 mmole) was treated with sodium hydride and stirred for 30 min at 0-4°C. 1-Chloro-1,2-dideoxy-3,5- di-O-toluoylribofuranose was added and the solution stirred for 1 hr at 0-4°C. The solution was poured into a saturated solution of sodium bicarbonate and extracted with dichloromethane. The organic layer was dried over sodium sulfate and evaporated to dryness. The residue was flash chromatographed on silica gel using toluene/ethyl acetate (5/1) as eluent. Two major products were isolated and identified as the N-1 and N-2 isomers in 57% (3.6 g) and 20% N-1 and N-2 (1.2 g) yields, respectively. Approximately 1 g of a mixture of N1 and N2 isomers was also collected. Overall yield of glycosylated material was 5.8 g (92%). The N1 isomer, 5-amino-1-(2-deoxy-3,5-di-O-toluoyl-β-D-erythro-pentofuranosyl)-3-[(5-tritylamino)pentyl]pyrazole-4-carbonitrile, was used without further purification in Example 6.

EXAMPLE 6:

1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamin-o)pentyl]pyrazolo[3,4-d]pyrimidin-4-amine.

To a toluene (100 mL) solution of the pyrazole-4- carbonitrile of Example 5 (3.5 g, 4.4 mmole) was added diethoxymethyl acetate (1.1 mL, 6.7 mmole). The solution was kept at 80-90°C for 5 hr and then evaporated to a syrup. The syrup was dissolved in dichloromethane (10 mL) and added to ice cold methanolic ammonia (100 mL) in a glass pressure bottle.

After two days at RT the contents of the bottle were evaporated to dryness. The residue was dissolved in methanol and adjusted to pH 8 with freshly prepared sodium methoxide to complete the deprotection. After stirring overnight the solution was treated with Dowex®-50 H+ resin, filtered, and evaporated to dryness. The residue was chromatographed on silica gel using acetone/hexane (3/2) as eluent to give 2.0 g (77%) of analytically pure product.

EXAMPLE 7:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-3-[5-(tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin-4-amine 5'-monophosphate.

To an ice cold solution of the pyrazolopyrimidin-4-amine of Example 6 (250 mg, 0.43 mmole) in trimethyl phosphate (5 mL) was added phosphoryl chloride (50 μL) and the solution kept at 0-4°C. The reaction was monitored by reversed phase HPLC using a linear gradient from 0 to 100% acetonitrile in water over 25 min. After stirring for 5 hr an additional aliquot of phosphoryl chloride (25 μL) was added and the solution stirred another 30 min. The solution was poured into 0.1M ammonium bicarbonate and kept in the cold overnight. The solution was then extracted with ether and the aqueous layer evaporated to dryness. The residue was dissolved in water (5 mL) and purified by reversed phase HPLC using a 22mm X 50cm C18 column. The column was equilibrated in water and eluted with a gradient of 0 to 100% acetonitrile over 20 min. Fractions containing the desired material were pooled and lyophilized to give 160 mg (56%) of chromatographically pure nucleotide.

EXAMPLE 8:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-3-(5-[(6-biotin-amido)hexamido]pentyl}pyrazolo[3,4-d]pyrimidin-4-amine 5'-monophosphate.

An ethanol solution (10 mL) of the nucleotide of Example 7, palladium hydroxide on carbon (50 mg), and cyclo- hexadiene (1 mL) was refluxed for 3 days, filtered, and evaporated to dryness. The residue was washed with dichloromethane, dissolved in DMF (1.5 mL) containing triethylamine (100 μL), and treated with N-hydroxysuccinimi- dyl biotinylaminocaproate (50 mg). After stirring overnight an additional amount of N-hydroxysuccinimidyl 6-biotinamidocaproate (50 mg) was added and the solution stirred for 18 hr. The reaction mixture was evaporated to dryness and chromatographed following the procedure in Example 7. Fractions were pooled and lyophilized to give 80 mg of chromatographically pure biotinamido-substituted nucleotide.

EXAMPLE 9:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-3-[5-(6-biotina-mido)hexamidopentyl]pyrazolo[3,4-d]pyrimidin-4-amine 5'-triphosphate.

The monophosphate of Example 8 (80 mg, ca. 0.1 mmole) was dissolved in DMF with the addition of triethylamine (14 μL). Carbonyldiimidazole (81 mg, 0.5 mmole) was added and the solution stirred at RT for 18 hr. The solution was treated with methanol (40 μL), and after stirring for 30 min tributylammonium pyrophosphate (0.5 g in 0.5 mL DMF) was added. After stirring for 24 hr another aliquot of tributylammonium pyrophosphate was added and the solution stirred overnight. The r action mixture was evaporated to dryness and chromatographed following the procedure in

Example 8. Two products were collected and were each separately treated with conc. ammonium hydroxide (1 mL) for 18 hr at 55°C. UV and HPLC analysis indicated that both products were identical after ammonia treatment and were pooled and lyophilized to give 35.2 mg of triphosphate nucleotide.

<u>EXAMPLE 10</u>:

NICK-TRANSLATION REACTION

The triphosphate of Example 9 was incorporated into pHPV-16 using the nick tanslation protocol of Langer et al. The probe prepared with the triphosphate of Example 9 was compared with probe prepared using commercially available bio-11-dUTP (Sigma Chemical Co). No significant differences could be observed in both a filter hybridization and in *in situ* smears.

More specifically, the procedure involved the following materials and steps:

Materials:

DNase (ICN Biomedicals) - 4ug/ml

DNA polymerase 1 (U.S. Biochemicals) - 8 U/ml

pHPV - 16 - 2.16 mg/ml which is a plasmid containing the genomic sequence of human papillomavirus type 16.

10X-DP - 1M Tris,pH7.5(20ml); 0.5M DTT(80 μl); 1M $MgCl_2$(2.8 ml); H_2O(17ml)

Nucleotides - Mix A - 2mM each dGTP, dCTP, TTP (Pharmacia)

Mix U - 2mM each dGTP, dCTP, dATP

Bio-11-dUTP - 1.0 mg/ml (BRL)
Bio-12-dAPPTP - 1.0 mg/ml

Steps:

To an ice cold mixture of 10X-DP(4μl), pHPV-16(2μl), nucleotide mix A (6 μl), Bio-12-dAPPTP(2μl), and H_2O(20μl) was added DNase (1μl) and DNA polymerase 1 (2.4μl). The reaction mixture was incubated at 16°C for 1 hr. The procedure was repeated using Bio-11-dUTP and nucleotide mix U in place of Bio-12-dAPPTP (comprising the triphosphate of Example 9) and nucleotide mix A.

Nucleic acid was isolated by ethanol precipitation and hybridized to pHPV-16 slotted onto nitrocellulose. The hybridized biotinylated probe was visualized by a streptavidin - alkaline phosphatase conjugate with BCIP/NBT substrate. Probe prepared using either biotinylated nucleotide gave identical signals. The probes were also tested in an in situ format on cervical smears and showed no qualitative differences in signal and background.

EXAMPLE 11:

5-Amino-3-[(5-tritylamino)pentyl]pyrazole-4-carboxamid-e.

Following the procedure of Example 2, except that cyanoacetamide is used instead of malononitrile, 5-(trityl- amino)pentylhydroxymethylenecyanoacetamide is prepared from 6-(tritylamino)caproic acid. This is then treated with diazomethane to give the methoxy derivative, following the procedures of Example 3, which is then reacted with hydrazine monohydrate, as in Example 4, to give 5-amino-3-[(5-tritylamino)pentyl]pyrazole-4-carboxamide.

EXAMPLE 12:

4-Hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyrazolo- [3,4-d]pyrimidine.

The carboxamide from Example 11 is reacted with potassium ethyl xanthate and ethanol at an elevated temperature to give the potassium salt of 4-hydroxypyrazolo- [3,4-d]pyrimidine-6-thiol. This salt is then reacted with iodomethane to give 4-hydroxy-6-methylthio-3-[(5-tritylamino)pentyl]pyrazolo[3,4-d]pyrimidine.

EXAMPLE 13:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-hydroxy-3-[5-(tritylamino)pentyl]pyrazolo[3,4-d]pyrimidin-6-amine.

Following the procedure of Example 5, the pyrazolopyrimidine of Example 12 is treated with sodium hydride and reacted with 1-chloro-1,2-dideoxy-3,5-di-O-toluoylribofuranose. The resulting compound is reacted with MCPBA and with methanolic ammonia, and the toluoyl protecting groups are removed to give the product.

EXAMPLE 14:

1-(2-Deoxy-β-D-erythro-pentofuranosyl)-4-hydroxy-3-[5-(6-biotinamido)hexamidopentyl]pyrazolo[3,4-d]pyrimidin--6- amine 5'-monophosphate.

Following the procedure of Example 7, the pyrazolopyrimidine of Example 13 is reacted with phosphoryl chloride to give the corresponding 5'-monophosphate.

Following the procedure of Example 8, the above 5'-monophosphate is reacted with palladium/carbon and cyclohexadiene, and the residue is reacted with N-hydroxy- succinimidyl biotinylaminocaproate to give

1-(2-deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-(6-biotinamido)hexamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine 5'-monophosphate.

EXAMPLE 15:

1-(2-Deoxy-β-D-erythropentofuranosyl)-4-hydroxy-3-[5-(-6-biotinamido)hexamidopentyl]pyrazolo[3,4-d]pyrimidin-6-amine 5'-triphosphate.

Following the procedure of Example 9, the 5'-mono- phosphate of Example 14 is treated with carbonyldiimidazole and then reacted with tributylammonium pyrophosphate to give the corresponding 5'-triphosphate.

EXAMPLE 16:

1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)pentyl]pyrazolo[3,4-d]pyrimidine-4-amine from Example 6 is reacted with benzoyl chloride and pyridine to give 1-(2-deoxy-3,5-di-O-benzoyl-β-D-erythropentofuranosyl)-3-[5-(tritylamino)pentyl] pyrazolo[3,4-d]pyrimidine-4-dibenzoylamine. This is treated with aqueous sodium hydroxide to partially deprotect the compound, giving 1-(2-deoxy-β-D-erythropentofuranosyl)-3-[5-(tritylamino)-pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

EXAMPLE 17:

1-(2-Deoxy-β-D-erythropentofuranosyl)-3-[5-(trifluoroacet- amido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

Following the procedure of Example 8, the benzoyl- amine of Example 16 is treated with palladium hydroxide on carbon and then with trifluoroacetic anhydride to give 1-(2-deoxy-β-D-erythropentofuranosyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine.

EXAMPLE 18:

1-(2-Deoxy-5-O-dimethoxytrityl-β-D-erythropentofuranosyl)-3-[5-(trifluoroacetamido)pentyl]pyrazolo[3,4-d]pyrimidine-4-benzoylamine 3'-O-(N,N-diisopropyl) phosphoramidite cyanoethyl ester.

The compound of Example 17 is reacted with dimethoxytrityl chloride and pyridine to give the corresponding 5'-dimethoxytrityl compound. This compound is then reacted with cyanoethyl chloro-N,N-diisopropylphosphor- amidite (according to the method of Sinha et al., Nucleic Acids Res. 12:4539 (1984)) to give the 3'-O-activated nucleoside.

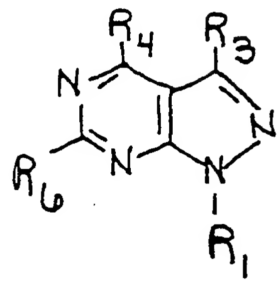
From the foregoing, it will be appreciated that the compositions of the present invention are novel and are useful for diagnostic purposes.

Although the present invention has been described in some detail by way of example for purposes of clarity and understanding, it will be apparent that other arrangements and equivalents are possible and may be employed without departing from the spirit and scope

of the invention. Therefore, the description and illustrations should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

WHAT IS CLAIMED IS:

1. A compound of the following formula (I):

(I) 

wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl;

n is zero or one; and

t is zero to twelve.

2. A compound according to Claim 1 wherein R_3 is the group $-W-(X)_n-A$, and A is a reporter group.

3. A compound according to Claim 2 wherein each of W and X is independently C_{1-12}alkylene, C_{1-12}alkenylene, C_{1-12}alkynylene, or such groups substituted at a terminal point with an oxy, thio, or amino, or a blocked derivative thereof.

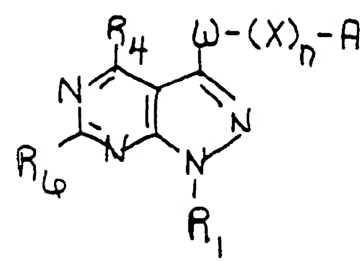
4. A compound according to Claim 1 wherein R_1 is a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation.

5. A compound according to Claim 2 wherein R_1 is a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation.

6. A compound according to Claim 1 wherein each of R_4 and R_6 is independently H, OH, or NH_2.

7. A compound according to Claim 1 wherein the sugar moiety is ribose, deoxyribose or arabinose.

8. A compound of the following formula (Ia):



(Ia)

wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation;

each of W and X is independently a chemical linker arm;

A is a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl;

n is zero or one; and

t is zero to twelve.

9. A compound according to Claim 8 wherein each of W and X is independently C_{1-12}alkylene, C_{1-12}alkenylene, C_{1-12}alkynylene, or such groups substituted at a terminal point with an oxy, thio, or amino or a blocked derivative thereof.

10. A compound according to Claim 8 wherein R_1 is a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation.

11. A compound according to Claim 10 wherein the sugar moiety is ribose, deoxyribose or arabinose.

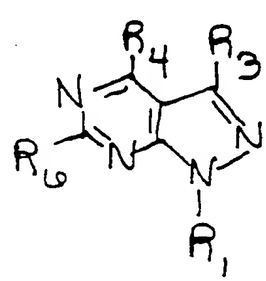
12. A compound according to Claim 8 wherein each of R_4 and R_6 is independently H, OH, or NH_2.

13. A compound according to Claim 10 wherein each of R_4 and R_6 is independently H, OH, or NH_2.

14. A compound according to Claim 8 wherein the reporter group is biotin.

15. A compound according to Claim 14 wherein the group $-W-(X)_n-$ is $-C_{1-12}$alkyl-NH-C(O)-C_{1-12}alkyl-NH-.

16. An oligonucleotide sequence which comprises at least one of the following:



(I)

wherein,

R_1 is a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation;

R_3 is hydrogen or the group $-W-(X)_n-A$;

each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl; and

t is zero to twelve.

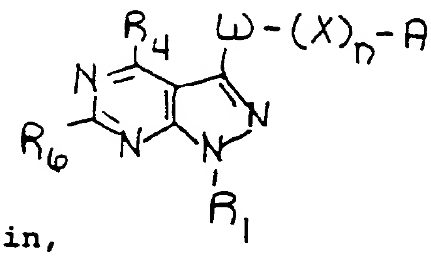
17. An oligonucleotide sequence according to Claim 16 wherein R_3 is the group $-W-(X)_n-A$, and A is a reporter group.

18. An oligonucleotide sequence according to Claim 17 wherein each of W and X is independently C_{1-12}alkylene, C_{1-12}alkenylene, C_{1-12}alkynylene, or such groups substituted at a terminal point with an oxy, thio, or amino or blocked derivative thereof.

19. An oligonucleotide sequence according to Claim 16 wherein each of R_4 and R_6 is independently H, OH, or NH_2.

20. An oligonucleotide sequence according to Claim 16 wherein the sugar moiety is ribose, deoxyribose or arabinose.

21. An oligonucleotide sequence which comprises at least one of the following:



(Ia)

wherein,

R_1 is a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation;

each of W and X is independently a chemical linker arm;

A is a reporter group;

each of R_4 and R_6 is independently H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

R is H or C_{1-6}alkyl;

n is zero or one; and

t is zero to twelve.

22. An oligonucleotide sequence according to Claim 21 wherein each of W and X is independently C_{1-12}alkylene, C_{1-12}alkenylene, C_{1-12}alkynylene, or such groups substituted at a terminal point with an oxy, thio, amino or blocked derivative thereof.

23. An oligonucleotide sequence according to Claim 21 wherein the sugar moiety is ribose, deoxyribose or arabinose.

24. An oligonucleotide sequence according to Claim 21 wherein each of R_4 and R_6 is independently H, OH, or NH_2.

25. An oligonucleotide sequence according to Claim 21 wherein the reporter group is biotin.

26. An oligonucleotide sequence according to Claim 25 wherein the group $-W-(X)_n-$ is $-C_{1-12}alkyl-NH-C(O)-C_{1-12}alkyl-NH-$.

27. A method for identifying target nucleic acid sequences, which method comprises utilizing an oligonucleotide probe including at least one labeled pyrazolo[3,4-d]pyrimidine of formula (Ia) as defined in Claim 8.

28. A method according to Claim 27 which comprises the steps of:

(a) denaturing nucleic acids in the sample to be tested;

(b) hybridizing to the target nucleic acids an oligonucleotide probe including at least one labeled substituted pyrazolo[3,4-d]pyrimidine of formula (Ia), wherein the probe comprises a sequence complementary to that of the target nucleic acids;

(c) washing the sample to remove unbound probe; and

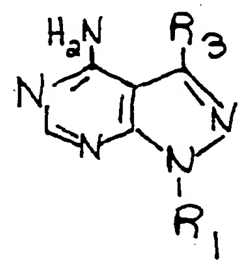
(d) detecting duplex formation between the target and probe nucleic acids.

29. An assay for identifying target nucleic acid sequences, which assay comprises utilization of an oligonucleotide probe including at least one labeled substituted pyrazolo[3,4-d]pyrimidine of formula (Ia) as defined in Claim 8.

30. A kit for identifying target nucleic acid sequences, which kit comprises a probe reagent component comprising an oligonucleotide including at least one labeled substituted pyrazolo[3,4-d]pyrimidine of formula (Ia) as defined in Claim 8, the oligonucleotide having a sequence complementary to that of the target nucleic acids; a denaturation reagent for

converting double-stranded nucleic acid to single-stranded nucleic acid; and a hybridization r action mixture.

31. A process for the synthesis of 3,4-disubstituted pyrazolo[3,4-d]pyrimidines of the following formula



wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

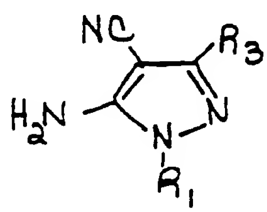
each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group; and

n is zero or one;

which process comprises

a) reacting a 3-substituted-5-aminopyrazole-4- carbonitrile of formula VI

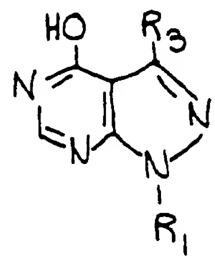


VI

wherein R_1, and R_3 are as defined above, with a dialkoxymethyl ester of a carboxylic acid at room temperature or above room temperature; and

b) reacting the resulting intermediate with ammonia.

32. A process for the synthesis of 3,4-disubstituted pyrazolo[3,4-d]pyrimidines of the following formula



wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

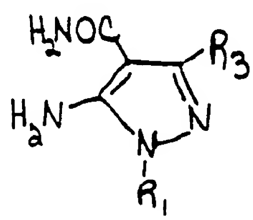
each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group; and

n is zero or one;

which process comprises

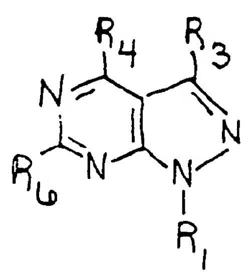
a) reacting a 3-substituted-5-aminopyrazole-4- carboxamide of formula VII



VII

wherein R_1, and R_3 are as defined above, with a dialkoxymethyl ester of a carboxylic acid at room temperature or above room temperature.

33. A process for the synthesis of 3,4,6-tri- substituted pyrazolo[3,4-d]pyrimidines of the following formula



wherein,

R_1 is hydrogen, or a sugar moiety optionally substituted at its 3' or its 5' position with monophosphate, diphosphate, triphosphate, or a reactive group suitable for nucleotide bond formation; provided that when R_3 is hydrogen then R_1 cannot be hydrogen;

R_3 is hydrogen or the group $-W-(X)_n-A$;

each of W and X is independently a chemical linker arm;

A is an intercalator, an electrophilic crosslinker or a reporter group;

R_4 is OH or NH_2;

R_6 is H, OH, SR, NH_2, or $NH(CH_2)_tNH_2$;

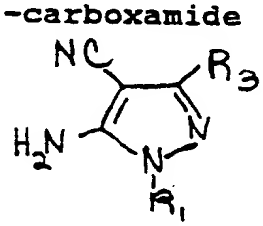
R is H or C_{1-6}alkyl;

n is zero or one; and

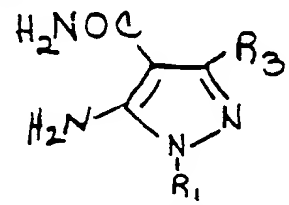
t is zero to twelve;

which process comprises

a) reacting a 3-substituted-5-aminopyrazole-4- carbonitrile of formula VI or a 3-substituted-5-aminopyrazole-4-carboxamide of formula VII



VI



VII

wherein, each of R_1, R_3, R_6, W, X, A, R, n and t are as defined above,

with an alkyl xanthate salt and with an alkyl halide, at a temperature above room temperature;

b) oxidizing the subsequent intermediate with a peroxide; and

c) where R_6 is other than -SR, reacting the subsequent intermediate with a group R_6-H.

34. The process of Claim 33 wherein R_6 is an amine.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/04184

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC 5th ed.: C07D 239/00; C07H 17/00; C07H 19/06; C07H 15/12;
US Cl.: 544/255; 536/24; 536/26; 536/27-29; 435/5-7

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US Cl.	544/255; 536/24; 536/26; 536/27-29; 435/5-7

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	N, "Helvetica Chimica Acta," Volume 71, Issued 1988, (Switzerland), F. Seela et al., "8-Aza-7-deazaadenine N^8- and N^8-(β-D-2'-Deoxyribofuranosides): Building Blocks for Automated DNA Synthesis and Properties of Oligodeoxyribonucleotides," see pp. 1813-1823.	1-26 27-30
X Y	N, "Helvetica Chimica Acta," Volume 71, Issued 1988 (Switzerland), F. Seela et al., "8-Aza-7-deaza-2'-deoxyguanosine: Phosphoramidite Synthesis and Properties of Octanucleotides," see pp. 1191-1198.	1-26 27-30
Y	N, "Nucleic Acids Research," Volume 14, Number 4, Issued 1986, IRL Press Limited (Oxford, England), F. Seela et al., "Phosphoramidites of base-modified 2'-deoxyinosine isosters and solid-phase synthesis of d(GCICGC) oligomers containing an ambiguous base," see pp. 1825-1844.	1-26

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 JAN 1990	06 FEB 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	L. Eric Crane

Form PCT/ISA/210 (second sheet) (Rev.11-87)

FURTHER INFORMATION CONTINUED FR M THE 2nd SHEET
(Not for publication)

C07H 17/00; C 12 Q 1/70; C12Q 1/68; G01N 53/00

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	EP, B 0,021,293, THE WELLCOME FOUNDATION LIMITED, Published 07 January 1981, see pp. 1-32 and claims 1-12.	1-26
Y	US, A 4,582,789 SHELDON ET AL., Published 15 April 1986, see columns 1-44	1-26
Y	US, A 4,711,955, WARD ET AL., Published 08 December 1987, see columns 1-34.	1-26

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers .... , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

3. ☐ Claim numbers ____________, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	N, "Chemical Syntheses and Transformations of Nucleosides," L. Goodman, Chapter 2 in "Basic Principles in Nucleic Acid Chemistry," Volume 1, Issued 1974, Academic Press (New York, New York), P. Ts'O ed., see pp. 116-117.	31, 32
Y	N, "Reagents for Organic Synthesis," volume 1, Issued 1967, John Wiley And Sons, Inc. (New York, New York), L. F. Fieser et al., see p. 837.	33, 34
Y	N, "Organic Chemistry of Nucleic Acids, Part B," Issued 1972, Plenum Press (New York, New York), see p. 375.	33,34
Y	US, A 3,598,807, NAKAYAMA ET AL., 10 August 1971, see columns 1-6.	1-15
Y	JP, A 61-109,797, YUKI GOSEI KOGYO LTD, Published 28 May 1986, see pp. 1047-1052; see also "Chemical Abstracts," vol. 106, Issued 1987 (Columbus Ohio), N. Sugimoto et al., "Labeled (poly)nucleotides," see abstract number 214304h.	1-26
Y	US, A 3,962,211, L. TOWNSEND ET AL., Published 08 June 1976, see columns 1-16.	1-15
A	N, "Biochemistry," Volume 15, number 5, Issued 1976, (Easton, Pennsylvania), S. Hecht et al., "Synthesis and Biological Activity of Pyrazolo[3,4-d]pyrimidine Nucleosides and Nucleotides Related to Tubercidin, Toyocamycin, and Sangivamycin," see pp. 1005-1015.	